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A Rapid and Easy Electrophoretic Method for Detecting Biochemical Loci of Rat (Rattus norvegices)

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Abstract: *Methods for electrophoresis for the analysis of biochemical marker genes, which are used widely for genetic monitoring of inbred straines of rats, have been complicated by the variation of the gel and electrode buffer and electrophoretic conditions with the enzyme or the protein to be examined. To simplify the methods, we performed electrophoresis under fixed conditions of 200 V and 30 min using cellulose acetate membrane as the gel and veronal solution as the gel and electrode buffer. Good results were obtained concerning 12 loci, namely, Amy1, Cs1, Esl, Es2, Es3, Es4, Fh1, Gc, Hbb, Ldr1, Mup1, and Svp1. This method was applied to 8 inbred strains of rats and confirmed to be practical.*

Key words: *biochemical gene, electrophoresis, rat*

Inbred mice have made great contributions to the development of research in life sciences, typically medicine and biology. On the other hand, rats, which are used in the largest number next only to mice, have not been employed often in the field of genetics, because more work and space are needed for their maintenance due to their greater size. As a result, their strains have not been as well developed as mice strains, but inbred rats have come to be used increasingly with the recent development of transplantation immunology, because rats have larger organs, are easier to handle, and provide serial samples including blood in larger amounts than mice.

Today 100–150 inbred rat strains are considered to have been established. Clarification of the genetic characteristics of each of these strains to a level similar to mice is extremely important for the future popularization of the use of rats in various fields. At the same time, a system for genetic monitoring for the follow-up of the characteristics of each strain after their clarification must also be established. At present genetic monitoring of rats is done primarily by methods developed for mice.

Among them, the method using biochemical marker genes, which is based on the polymorphism of proteins and enzymes, is used most frequently,but the electrophoretic method reported for this purpose was complicated because the gel, buffer, time and other electrophoretic conditions varied with the target protein or enzyme.

In this study, we examined the biochemical marker genes of 12 proteins and enzymes by a simple electrophoretic method, in which a commercially available cellulose acetate membrane is used as the gel, and the

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buffer, voltage, and running time are uniform, in inbred strains of rats maintained at the Institute for Experimental Animals, Hamamatsu University School of Medicine. The results were excellent.

Eight strains, namely ACI/Ham, BN/Ham, DA/Ham, IS/Ham, LOU/M/Ham, PVG/c/Ham, NIG-III/Ham and TM/Ham, were examined.

Blood was collected in vessels containing an anticoagulant and red blood cells and plasma were separated by centrifugation at 3,000 rpm for 15 min.

Plasma was frozen at −20°C. Red blood cells were washed 3 times with physiologic saline and hemolyzed with the same volume of distilled water by microplate. Since fresh hemolysate is needed for electrophoresis of the hemoglobin beta chain (*Hbb*), part of the hemolyzed blood was stored at 4°C and analyzed within 2–3 days.

The hemolysate used for electrophoresis of enzymes was stored at −20°C. For the analysis of catalase (*Cs1*), however, the above hemolysate frozen at −20°C was thawed within 1 week, and diluted 4–5 times.

Urine was pooled overnight in a metabolic cage or was collected directly from the bladder. A clearer zymogram pattern was obtained by diluting the pooled urine 100–200 times. The sample was gently centrifuged and the supernatant was frozen at −20°C.

Seminal vesicle glands were carefully separated and the fluid was mixed with the same volume of physiologic saline. Diluted seminal vesicle fluid was centrifuged at 12,000 rpm for 10 min at 4°C, and the supernatant was stored at −20°C until use.

The small intestine, kidney and pancreas were homogenized with two weights of distilled water, the homogenates were centrifuged at 12,000 rpm for 10 min at 4°C, and the supernatants were stored at −20°C.

Veronal buffer (pH 8.6, hereafter refered to simply as "buffer") alone was used for both bufferization of the cellulose acetate (CA) membrane and electrophoresis. This buffer was prepared by dissolving 10.3 g of Na-diethyl barbiturate and 1.8 g of diethyl barbiturate with 1,000 m*l* of distilled water.

Titan-III CA memblane (Cat. No. 3023) sold by Helena Research Laboratory, Ltd. (Beaumont, TEXAS, USA) was used as the gel for electrophoresis of both proteins and enzymes [2]. CA membranes were immersed in the buffer for about 10 min prior to electrophoresis.

Sample wells (Cat. No. 4085) to hold divisions of the sample, an applicator (Cat. No. 4084) to apply the sample, and an aligning base (Cat. No. 4086) to immobilize the CA membrane were prepared for smearing the samples.

A thawed sample was poured into the sample groove of the sample well at 8 μ*l*/well, and the groove was covered with a glass plate to prevent the sample from evaporating.

The CA membrane soaked with the buffer was wiped with filter paper to remove unnecessary moisture, placed on an aligning base, and smeared with a sample.

The sample was smeared on the anode side for examination of most items but in the center for the analysis of *Amy1*, *Ldr1*, and *Svp1*.

Electrophoresis was performed at 200 V (6 mA/gel) for 30 min for all proteins and enzymes.

Table 1 shows the 12 proteins or enzymes examined, names of thier loci, organs in which they were detected and the reagents used for their staining [1].

For staining of proteins (*Gc*, *Hbb*, *Mup1*, and *Svp1*), the CA membrane after electrophoresis was immediately immersed in a staining solution prepared by dissolving Ponceau-S in 0.5% tricholoracetic acid for 1 min and then immersed in 5% acetic acid for decoloration and fixation.

If bands could be clearly identified, the membrane was washed with water and air-dried.

Enzymes were stained either by immersing the CA membrane in a staining solution or by applying a mixture of the staining solution and agar solution to the CA membrane.

Amy1 was stained by immersing the CA membrane in 1.0% hydrolyzed starch solution for 10 min at room temperature and then in a solution of 1 g of iodine and potassium iodine in 300 m*l* of distilled water. *Cs1* was stained by immersing the CA membrane after electrophoresis in 0.03% hydrogen peroxide for 1 min and then in a mixture of 1.7% iron III chloride solution and 1.0% potassium ferricyanide solution prepared immediately before use.

For the analysis of other enzymes, the reagents shown in Table 1 were scaled in the indicated amounts per CA membrane and were dissolved with 2.5 m*l* of distilled water. At this time, agar was dissolved completely with distilled water at 0.8% in a microwave oven, 2.5 m*l* of this solution was quickly mixed with the reagent

Table 1. Tissue sample and staining method for 12 loci of protein and enzyme

Protein & Enzyme	Locus	Sample	Staining	
Amylase1	*Amy1*	Pancreas	Starch Hydrolysed	
			Iodine	
			Potassium iodine	
Catalase1	*Cs1*	RBC lysate	Hydrogen peroxide	
			Ferric chloride	
			Potassium ferricyanide	
Esterase1	*Es1*	Jejunum	β-naphtyl acetate	0.25 m*l*
			Fast blue RR	10 mg
Esterase2	*Es2*	Plasma	β-naphtyl acetate	0.25 m*l*
			Fast blue RR	10 mg
Esterase3	*Es3*	Jejunum	β-naphtyl acetate	0.25 m*l*
			Fast blue RR	10 mg
Esterase4	*Es4*	Kidney	β-naphtyl acetate	0.25 m*l*
			Fast blue RR	10 mg
Fumarate hydratase	*Fh1*	Liver	Fumaric acid	30 mg
			NAD	10 mg
			Na-pyruvate	25 mg
			MTT	3 mg
			PMS	1 mg
			Malate dehydrogenase	37.5 unit
Group-specific component	*Gc*	Plasma	Ponceau-S 0.5% in 5% Trichloro acetic acid	
Hemoglobin beta chain	*Hbb*	RBC lysate	Ponceau-S 0.5% in 5% Trichloro acetic acid	
Lactate dehydrogenase1	*Ldr1*	RBC lysate	NaDL-lactate	1.0 m*l*
			NBT	2 mg
			PMS	1 mg
			NAD	2 mg
Major urinary protein1	*Mup1*	Urine	Ponceau-S 0.5% in 5% Trichloro acetic acid	
Seminal vesicle protein1	*Svp1*	Seminal vesicle secretion	Ponceau-S 0.5% in 5% Trichloro acetic acid	

solution, and the mixture was layered over the entire surface of the CA membrane after electrophoresis. Since coloring began as soon as the mixture was layered, it was not necessary to maintain the temperature at 37°C, but the CA membrane was placed in a moisture box (the membrane was placed on wet filter paper in a container of an appropriate size) to prevent drying of the agar.

Coloring usually became sufficient for evaluation within 30 min, although this time varied among enzymes. The layered agar was removed, and the membrane was fixed and dried similarly to the analysis of protein.

Fig. 1 shows the zymogram patterns of the proteins and enzymes at the 12 loci and their genotypes in inbred strains of rats. And Figs. 2–5 show the zymogram patterns of *Cs1*, *Es1*, *3 Es4* and *Svp1*, respectively. Active bands of all proteins and enzymes examined could be clearly identified in the zymogram patterns. We tried to detect *Akp1*, *Alp1*, *Acon2* and *Acp2* but were not successful. Table 2 shows the results of this electrophoretic analysis in inbred strains of rats maintained at the Institute for Experimental Animals, Hamamatsu University School of Medicine.

The composition of the 12 loci in the 8 strains examined varied among the strains, and none of the strains showed an identical compositions. Four alleles, namely, *a*, *b*, *c*, and *d*, were observed for the gene locus *Es3*, and 2 alleles were observed in all the remaining 11 loci. Genotypes at *Gc* and *Es3* were characteristic of

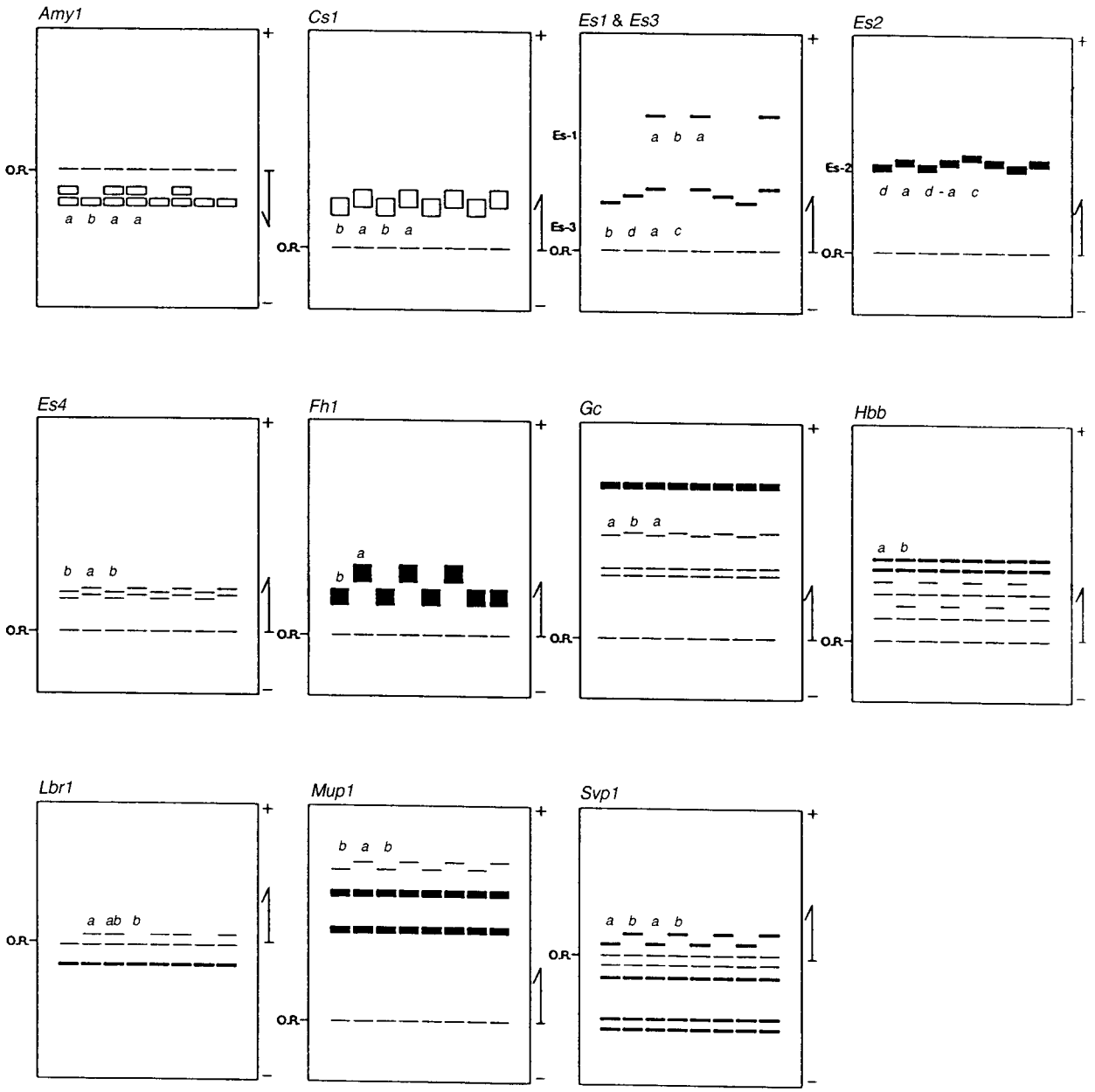

Fig. 1. Schematic illustrations of zymogram patterns of 12 loci (O.R.=Origin).

the TM/Ham strain, those at *Es2* and *Es3* of the NIG-III/Ham strain, and those at *Es3* and *Es4* of the IS/Ham strain.

In the development of the present electrophoretic system, we placed the priority on the simplicity and uniformity of the procedure from sample preparation to electrophoresis. Our method, in which a commercial CA membrane is used, eliminated the need to change the gel, buffer or electrophoretic conditions for different evaluation items. It is considered that this system can be easily run, even by investigators and students who have little experience in electrophoresis.

Acknowledgments

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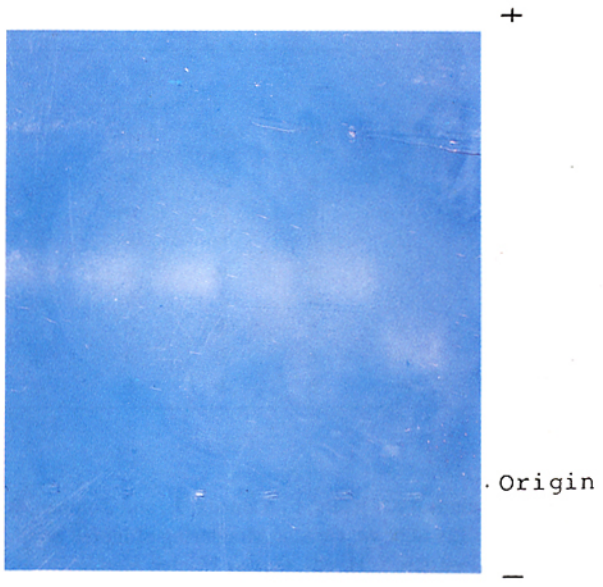

Fig. 2. Zymogram pattern of *Cs1*.

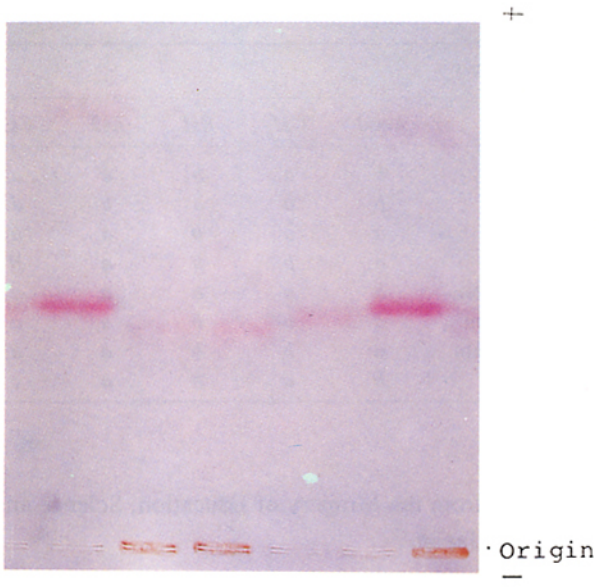

Fig. 3. Zymogram pattern of *Es1* and *Es3*.

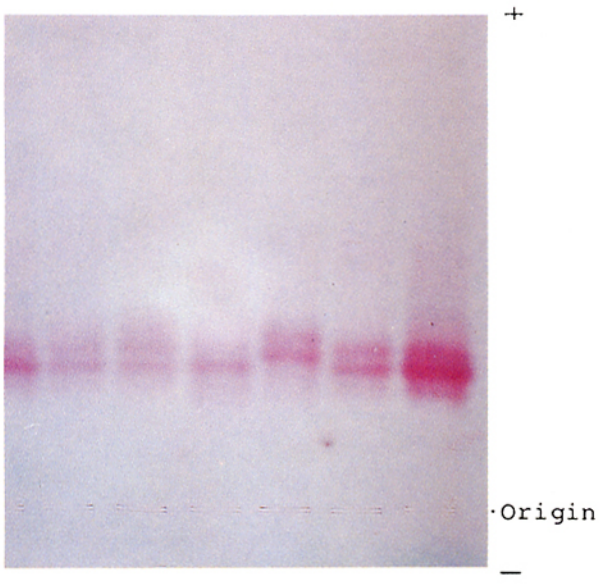

Fig. 4. Zymogram pattern of *Es4*.

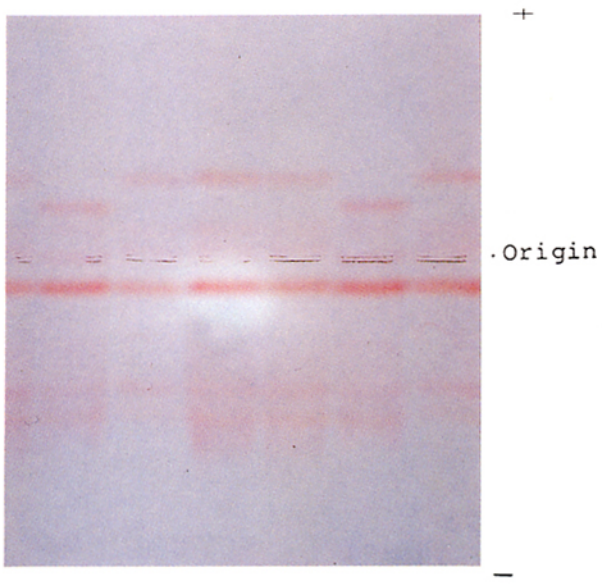

Fig. 5. Zymogram pattern of *Svp1*.

Table 2. Strain distribution patterns at 12 loci in 8 strains of rats

	Locus											
Strain	*Amy1*	*Cs1*	*Es1*	*Es2*	*Es3*	*Es4*	*Fh1*	*Gc*	*Hbb*	*Ldr1*	*Mup1*	*Svp1*
ACI/Ham	*b*	*a*	*b*	*a*	*a*	*b*	*b*	*a*	*b*	*a*	*b*	*a*
BN/Ham	*b*	*a*	*a*	*c*	*d*	*b*	*a*	*a*	*a*	*b*	*a*	*b*
DA/Ham	*a*	*a*	*b*	*a*	*a*	*b*	*b*	*a*	*b*	*b*	*b*	*a*
IS/Ham	*a*	*b*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*a*	*b*
LOU/M/Ham	*a*	*a*	*a*	*a*	*a*	*b*	*b*	*a*	*a*	*b*	*b*	*a*
PVG/c/Ham	*a*	*b*	*b*	*a*	*a*	*b*	*a*	*a*	*b*	*b*	*a*	*a*
NIG-III/Ham	*a*	*b*	*b*	*a*	*a*	*b*	*b*	*a*	*b*	*b*	*b*	*a*
TM/Ham	*b*	*a*	*b*	*a*	*c*	*b*	*b*	*b*	*b*	*b*	*b*	*a*

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